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Toxicological effects involved in risk assessment of rare earth lanthanum on roots of *Vicia faba* L. seedlings

Chengrun Wang[1,*], Mei He[1], Wen Shi[3], Jessie Wong[4], Tao Cheng[1], Xiaorong Wang[2], Lingling Hu[1], Fenfen Chen[1]

1. School of Life Science, Huainan Normal University, Huainan 232001, China. E-mail: chengrunw66@yahoo.com.cn
2. State Key Laboratory of Pollution Control and Resources Reuse, School of the Environment, Nanjing University, Nanjing 210046, China
3. Department of Chemical and Biochemical Engineering, University of Western Ontario, London, ON, N6A5B9, Canada
4. Department of Biology, University of Western Ontario, London, ON, N6A5B9, Canada

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Abstract

Combined chemical analyses and biological measurements were utilized to investigate potential toxicological effects and possible mechanisms involved in risk assessment of rare earth elements (REEs) on *Vicia faba* L. seedlings, which were hydroponically cultivated and exposed to various concentrations of lanthanum (La) for 15 days. The results showed that La contents in both the solution and roots increased with the increase of extraneous La, contributing to hormetic dose responses of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and endoprotease (EP) isozymes activities, and HSP 70 production enhanced at low doses but suppressed at higher doses of La. These physiological responses constituted antioxidant and detoxification systems against La-induced oxidative stress. The elevated La levels also contributed to oxidatively modified proteins, which were most responsible for subsequent cell death and growth retardation of the roots. By combination of hormetic and traditional threshold dose levels, the threshold dose range was deduced to be 108–195 μg La/g dry weight in the roots, corresponding to 0.90–3.12 mg/L of soluble La in the culture solution. It suggests that persistent applications of REEs may lead to potential ecological risk in the environment.

Key words: rare earth elements; lanthanum; heat shock protein 70; oxidatively modified proteins; isoenzymes; hormesis; risk assessment

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Introduction

Over the past thirty years, applications of rare earth elements (REEs) have been rapidly expanded in industrial, agricultural and forestry sectors as well as animal husbandry. Owing to their specific properties, REE-based micro-nutrient fertilizers, containing mainly cerium (Ce), lanthanum (La) and neodymium (Nd), are widely studied and applied for yield enhancement and quality improvement of certain crops and vegetables (Liu et al., 2006; Wang et al., 2004, 2007). REEs were also released into the environment from mining activities (Goetz et al., 1982). Thus, more and more REEs have entered the environments through anthropogenic activities.

However, as nonessential elements in organisms, the effects of REE accumulation on organisms remain fragmentary and inconsistent. Furthermore, the toxicological mechanisms and related environmental risk of persistent applications of REEs in agriculture remain unclear.

REEs have been confirmed to exert hormetic effects on plants (Ouyang et al., 2003). Hormesis is often observed in organisms under studies designed to assess responses below no observed adverse effect levels. One common conclusion is that this could cause seemingly beneficial effects (Calabrese and Baldwin, 2003a, 2003b), which may justify the application of REEs in agriculture. Accurate descriptions of hormetic dose-response curves help to determine efficacy and hazards of pollutants with hormetic phenomenon (Qin et al., 2010; Wang et al., 2010). Thus, hormetic effects and their related modes of action by REEs ought to be considered in ecological risk assessment of application of REEs in agriculture.

The seedlings of *Vicia faba* L. are widely cultivated in the world, which are also used as model tested plant species in environmental sciences. In this study, *V. faba* seedlings were employed as the tested materials and lanthanum nitrate ($La(NO_3)_3$) was used as a representative of light rare earth elements. The objectives were to gain insight into the toxicological effects and underlying mechanisms of La on the roots and provide data for further

* Corresponding author. E-mail: chengrunw66@yahoo.com.cn

ecological risk assessment regarding the applications of REEs in agriculture.

1 Materials and methods

1.1 Main reagents

Mouse monoclonal anti-dinitrophenyl IgE isotope and goat anti-mouse IgE conjugated with horseradish peroxidase were purchased from Sigma Chemical (St. Louis, MO, USA). Mouse anti-HSP 70/HSC 70 monoclonal antibody (SPA-820) and goat anti-mouse IgG conjugated with horseradish peroxidase were obtained from Stressgen Corp. (Ann Arbor, MI, USA). SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, USA), PageRuler^{TM} prestained protein ladders (Fermentas, Canada). Dithiothreitol, bovine serum albumin, nitro blue tetrazolium, 2,4-dinitrophenylhydrazine (DNPH), trifluoroacetic acid (TFA), Coomassie Brilliant Blue R-250, Triton X-100, $La(NO_3)_3$ and phenylmethanesulfonyl fluoride were obtained from Shanghai Chemical Reagent (Shanghai, China). All chemicals were of analytical grade and Milli-Q water was used throughout the experiments.

1.2 Plant material and La treatment

Seeds of *V. faba* were sterilized with 0.1% (*W*/*V*) sodium hypochlorite solution for 10 min and rinsed thoroughly in distilled water. Five uniformly germinated seeds were selected and transferred to a 1.2-L container filled with Hoagland solution (Lucretti et al., 1999). One mmol/L of ammonium dihydrogen phosphate was directly sprayed onto the seedlings every day. $La(NO_3)_3$ was dissolved in the solutions to achieve the desired concentrations of extraneous La of 0, 0.25, 0.5, 1, 2, 4, 8 and 12 mg/L, respectively. The solutions were replaced every day and pH values were maintained between 6.1 and 6.3. The seedlings were cultivated under controlled conditions (15-hr photoperiod with active radiation of 220 μmol/(m^2·sec), 80% relative humidity, and 23°C/20°C day/night regime) and aerated consecutively. Three parallels of containers were prepared in each treatment in two independent experiments. Roots were harvested for chemical analysis and biological measurements after exposure of 15 days.

1.3 Measurement of La contents in roots and culture solutions

The roots were subsequently rinsed with 1 mol/L HCl and then distilled water. Digestion of dried samples was performed according to methods of Wang et al. (2008a, 2008b). La contents were detected by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 5300DV, Perkin-Elmer, USA) and expressed as μg/g dry weight (dw). Certified standard samples (GBW07429) were used to ensure accuracy and precision. All results were detected above detection limits (0.02 μg/L). To determine the contents of soluble La in culture solutions, aliquots of the solutions were filtered through 0.22-μm-pore-size filters and the filtrate was directly determined by ICP-OES.

1.4 Determination of root lengths and cell death *in situ*

Root lengths were measured from stem base to primary root tips. Cell death was detected according to method described by Romero-Puertas et al. (2004b), magnified 50 times and photographed with microscope (Leica, BX51, Japan). The stained root tips were sliced transversely with a fresh razor blade to define the distribution of dead cells (magnified 100 times).

1.5 Determination of antioxidant isoenzymes and activities

Crude enzyme extracts were prepared according to method described by Romero-Puertas et al. (2004a). One gram of fresh roots was ground immediately into fine powder and homogenized in extraction buffer. The extracts were centrifuged at 15,000 ×*g* for 25 min, and soluble protein contents in supernatant were determined by method of Bradford (1976). All operations were performed at 4°C.

Isozyme patterns were determined by native polyacrylamide gel electrophoresis (PAGE) using high-throughput Mini-PROTEIN 3 electrophoresis system (Bio-Rad, USA). Electrophoresis was performed at constant voltage of 80 V reaching resolving gel, and constant voltage of 120 V to the end at 4°C using a 25 mmol/L Tris, 192 mmol/L glycine solution (pH 8.3) as running buffer.

Superoxide dismutase (SOD), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) isozymes were visualized according to method of García-Limones et al. (2002). Catalase (CAT) isozymes were visualized according to Verma and Dubey (2003). Integrated intensities of bands were quantified by Image J software (National Institutes of Health, USA) according to methods described by Barabás et al. (2000) and Biemelt et al. (2000) and normalized to controls, denoting respective activities.

1.6 SDS-PAGE and Western blotting of HSP 70

SDS-PAGE and Western blotting of HSP 70 were conducted according to methods of Wang et al. (2008a, 2008b) with minor modification. Constant weight of total proteins together with PageRuler^{TM} prestained protein ladders were separated by 10% SDS-PAGE. Total proteins in gels were transferred by Semi-Dry Transfer System (Bio-Rad, USA) onto PVDF membrane. The membranes were blocked with 8% non-fat milk/TBST buffer (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, containing 0.05% Tween-20) for 2 hr. After washing with TBST buffer, mouse anti-HSP 70/HSC 70 monoclonal antibody (1:5000) was added to the membranes and incubated at 4°C for 8 hr. Next, the membranes were incubated in anti-mouse IgG conjugated with horseradish peroxidase (1:10,000) at room temperature for 1 hr. Bands were visualized by the enhanced chemiluminescence and exposed to X-ray film (Shenbei, Shanghai, China). Integrated intensities of bands were quantified by Image J software and normalized to controls, denoting respective HSP 70 levels.

1.7 Immunoblotting of carbonyl proteins

Carbonyl proteins were detected according to method of Romero-Puertas et al. (2004a) with minor modification.

Crude enzyme extract of 50 μL was mixed with 50 μL of 10% (*W*/*V*) SDS and 100 μL of 20 mmol/L DNPH prepared in 20% (*W*/*V*) TFA. One hundred μL of 20% TFA instead of 20 mmol/L DNPH was added in a blank lane. All treatments were allowed to settle at room temperature for 20 min, followed by addition of 100 μL of 2 mol/L Tris buffer (pH 8.0), containing 30% (*V*/*V*) glycerol and 6% (*V*/*V*) β-mercaptoethanol, and 50 μL of 1% (*W*/*V*) bromophenol blue. In each treatment, 6.11 μg of total protein was subjected to 10% SDS-PAGE and Western blotting as described above. Mouse anti-dinitrophenyl IgE isotope (1:2000) and goat anti-mouse IgE (1:40,000) were used to detect the DNPH derivatives.

1.8 Determination of proteolytic activity of oxidized proteins

Endoproteinase (EP) isozymes were detected according to method introduced previously by Distefano et al. (1997) and Rui and Xu (2003). Constant weight of soluble protein in each treatment was subjected to 10% SDS-PAGE containing 0.2% (*W*/*V*) gelatin by high-throughput Mini-PROTEIN 3 Electrophoresis System. After electrophoresis, gels were transferred to Na-acetate buffer (pH 4.0) at 38°C for 2 hr to remove SDS, followed by incubation with 250 mmol/L Tris-HCl (pH 7.5) at 37°C for 3 hr. The gels were stained with 0.1% (*W*/*V*) Coomassie Brilliant Blue R-250 for 1 hr, and then destained in 50% methanol and 10% acetic acid. EP isozymes were visualized on dark-blue background as white bands. Integrated intensities of bands were measured by Image J software and normalized to controls, denoting respective activities.

1.9 Statistical analysis

All the statistical analyses were performed using SPSS 13.0 for Windows (SPSS, Chicago IL, USA). The data were all presented as the mean ± standard deviations of 3 replicates. Difference was considered to be significant at $p < 0.05$ (*) and highly significant at $p < 0.01$ (**) using one-way ANOVA by Dunnett's *t*-test. Representative photographs from the treatments were also presented.

2 Results

2.1 La contents in culture solution and root tissues

In root tissues, La contents increased with the increasing concentrations of extraneous or soluble La in the culture solution. Significant enhancement of La contents in the roots was shown when the extraneous La increased to be more than 1 mg/L (Fig. 1).

2.2 Effects of La on root tip cell death and root lengths

The root lengths decreased below the control at all treatments (Fig. 2a). Noticeably, the root lengths declined to be the shortest at 50.3 μg La/g dw root except at 433 μg La/g dw (lane 2) in all the treatments. The degree of cell death was slightly reduced at 74.9 μg La/g dw, but markedly enhanced at more than 232 μg La/g dw in the roots (Fig. 2b). Moreover, the transversely sliced slides showed that dead cells were mainly centralized in epidermal phloem (Fig. 2c).

2.3 Effects of La on patterns and activities of antioxidant enzymes

The results showed that no obvious changes were observed in the band number of SOD, CAT or APX patterns, except for varying intensities due to the increasing La (Fig. 3a, b and d). However, distinct changes were detected in GPX isozymes. For instance, the band 2 in the GPX patterns became blurry at 74.9–232 μg La/g dw (lanes 3–7) in the roots (Fig. 3c).

Relative activities of SOD initially increased from 10.2 to 74.9 μg La/g dw in the roots, tended to decline from 74.9 to 151 μg La/g dw, and then enhanced thereafter, showing a biphasic dose response curve (an inverted U-shaped curve, followed consecutively by a U-shaped or J-shaped dose response curve) (Fig. 4a). Similar biphasic curves were also showed in the activities of CAT and APX isozymes

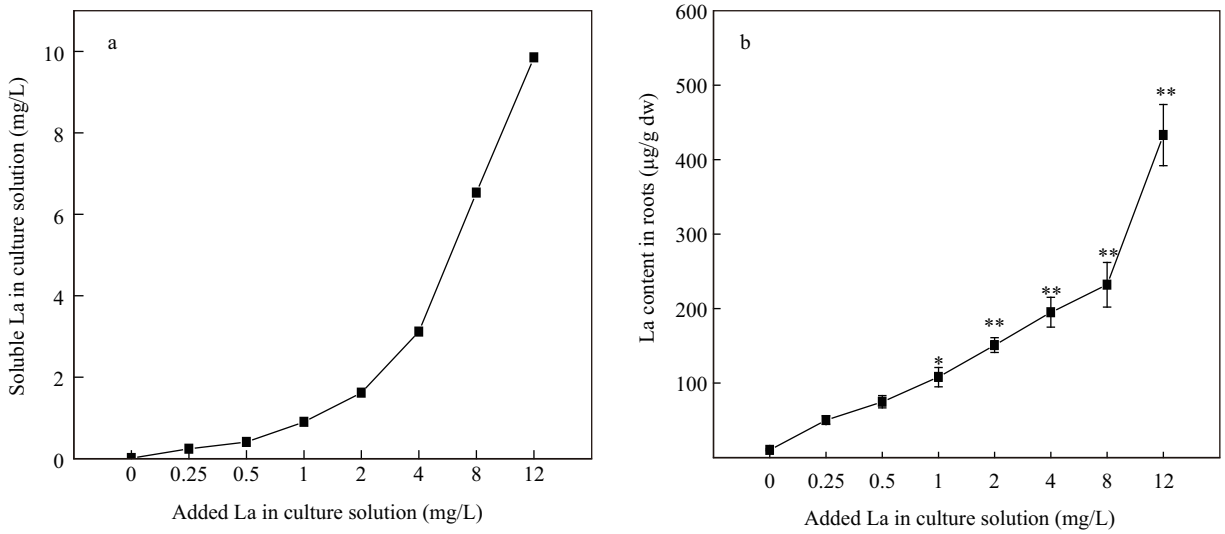

Fig. 1 Soluble La in culture solution (a) and La content in roots (b) of *V. faba* L. seedlings hydroponically cultivated in 0–12 mg/L of extraneous La (corresponding to 10.2, 50.3, 74.9, 108, 151, 195, 232 and 433 μg La/g dw root, respectively) for 15 days. Data are presented as mean in (a) and as mean ± SD (n= 3) in (b). *$p < 0.05$, **$p < 0.01$.

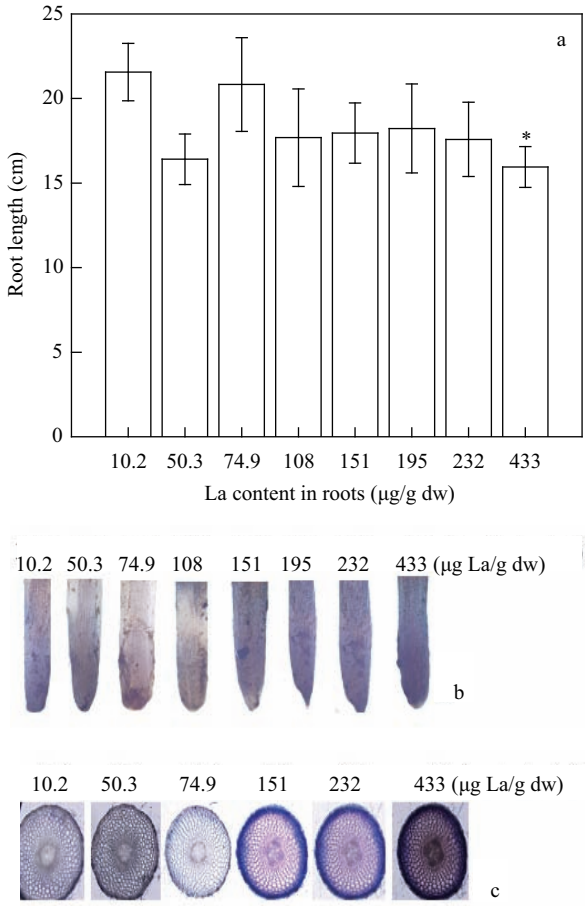

Fig. 2 Alteration in root lengths (a), cell death degrees (b) and cell death distribution (c) in root tips of *Vicia faba* L. seedlings hydroponically cultivated in 0–12 mg/L of extraneous La for 15 days. Evans Blue staining of root tips was indicative of cell death (50×). The transversely sliced slides showed the main distribution of dead cells in the tips (100×). Values are denoted as mean ± SD (n = 3). * $p < 0.05$.

(Fig. 4b and d). However, only a single U-shaped curve was observed in the GPX activities (Fig. 4c).

2.4 La-induced HSP 70 production

HSP 70 production was detected by Western blotting (Fig. 5a). The result showed that the HSP 70 levels were enhanced above the control at 10.2 to 74.9 μg La/g dw in the roots, and then tended to decline, which exhibited an inverted U-shaped curve. HSP 70 was significantly decreased when the contents of La increased up to 195 μg La/g dw in the roots (Fig. 5b).

2.5 La-induced oxidatively modified proteins

The antibody's specificity was verified by the blank without DNPH treatment, showing almost no blot (Fig. 6b). The intensities of bands reached a peak value at 50.3 μg La/g dw (lane 2) in the roots, and then tended to decrease until 195 μg La/g dw (lane 6), followed by enhancement (Fig. 6b). The intensities did not show any correlation with the increasing concentrations of La in the roots.

2.6 La-induced proteolytic activity of oxidized proteins

In patterns of EP isozymes, two bands were detected in all the treatments (Fig. 7a). The results showed that the relative activities of EP isozymes slightly decreased from 10.2 to 74.9 μg La/g dw in the roots, and enhanced thereafter, showing a U-shaped dose response curve (Fig. 7b).

3 Discussion

No studies demonstrated that REEs are essential elements in organisms including plants. However, increasing amounts of REEs are being unintentionally released into the environments with continuously increasing ap-

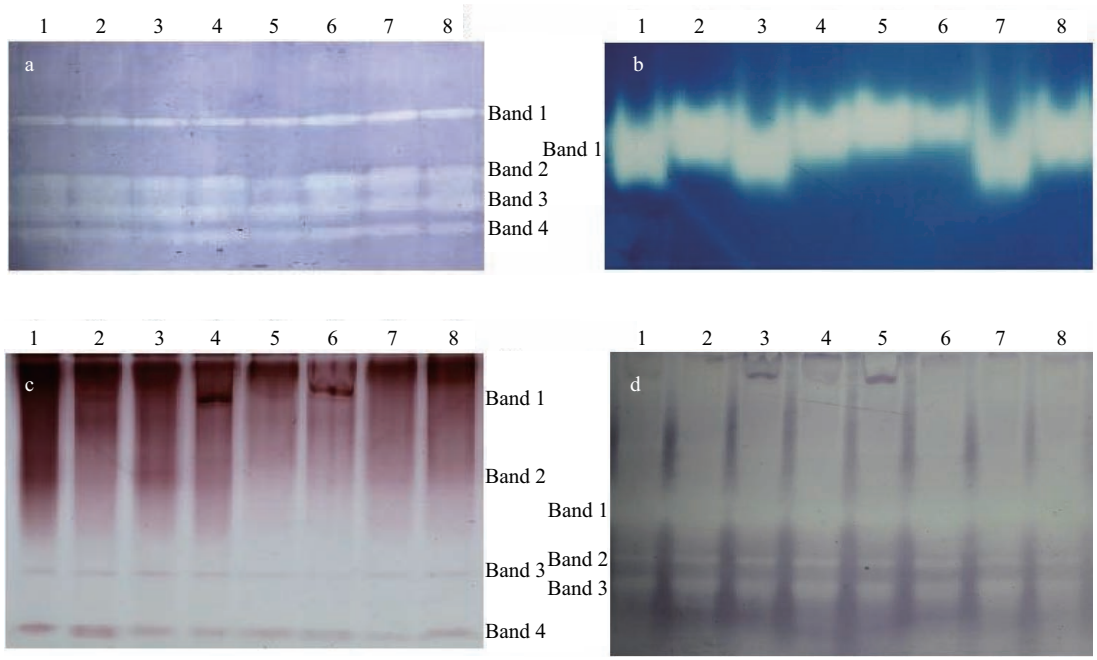

Fig. 3 Patterns of SOD (a), CAT (b), GPX (c), and APX (d) isozymes in roots of *V. faba* seedlings hydroponically cultivated in 0–12 mg/L of extraneous La for 15 days. Lanes 1–8 represent 10.2, 50.3, 74.9, 108, 151, 195, 232 and 433 μg La/g dw roots, respectively.

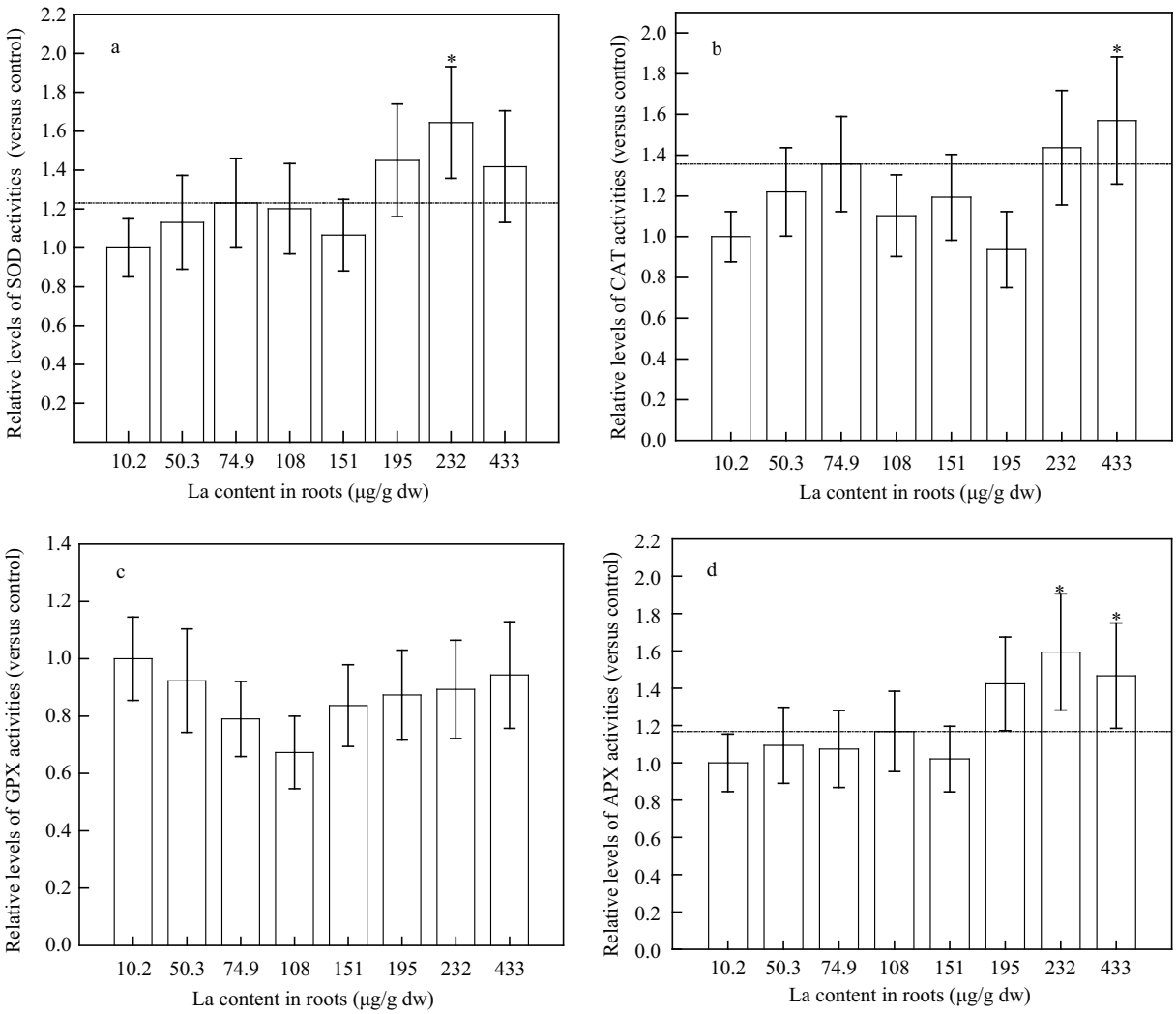

Fig. 4 Relative activities in SOD (a), CAT (b), GPX (c) and APX (d) isozymes in roots of *V. faba* seedlings cultivated hydroponically in 0–12 mg/L of extraneous La (corresponding to 10.2, 50.3, 74.9, 108, 151, 195, 232 and 433 μg La/g dw root, respectively) for 15 days. Data are presented as mean ± SD ($n = 3$). *$p < 0.05$. Short dash lines are introduced in the figures, proposed as a hormetic threshold level, above which represents oxidative stress.

plications of REEs and related mining activities. The toxicological effects, mechanisms and environmental risk assessment of persistent application of REEs in agriculture are not completely understood and need to be further investigated.

REEs, as heavy metals, are mainly absorbed through plant roots and tend to accumulate specifically in the roots. The present results showed that the increasing concentrations of extraneous La led to the increases of La contents in culture solution and root tissues (Fig. 1). The increased La in the roots was responsible for the elevation of antioxidant isozymes and corresponding activities in the roots (Figs. 3 and 4). Ascorbate, glutathione and APX activities were induced by La and REEs nitrate treatments in roots of *Triticum durum*, indicating oxidative stress (d'Aquino et al., 2009). Thus, in this study, the increasing activities of SOD, CAT, GPX and APX isozymes suggest that reactive oxygen species (ROS) were possibly produced and involved in the subsequent oxidative damage in the roots, especially under higher concentrations of La. ROS can also act as signals to induce defense response in organisms. These activated antioxidant isozymes also indicate that an elevated capability of ROS scavenging might alleviate oxidative stress due to La accumulation in the roots.

ROS were also proved to be positively correlated with HSP 70 induction (Gupta et al., 2005). HSP 70 is involved in repairing or accelerating degradation of denatured proteins, homeostatic maintenance and adaptation to various stressors. As shown in the results, the increase of La contents caused the enhancement of HSP 70 synthesis probably through the mediation of ROS in the roots. Thus, HSP 70 induction worked together with the antioxidant enzymes and constituted intracellular defense systems against La stress in the roots.

HSP 70 induction was used as a general biomarker of environmental stressors, such as heavy metal toxicity for terrestrial and aquatic animals (Arts et al., 2004; Hallare et al., 2005). However, HSP 70 has been less reported in plants compared with those in animals (Hildebrandt et al., 2007; Rivera-Becerril et al., 2005; Saidi et al., 2007; Wang et al., 2008a, 2008b). In this study, the inverted U-shaped dose response curve of HSP 70 levels suggest that HSP 70 induction can also be used as bioindicator of oxidative stress due to La stress in the roots.

ROS attack biomolecules and lead to lipid peroxidation, protein oxidation and DNA damage, ultimately resulting

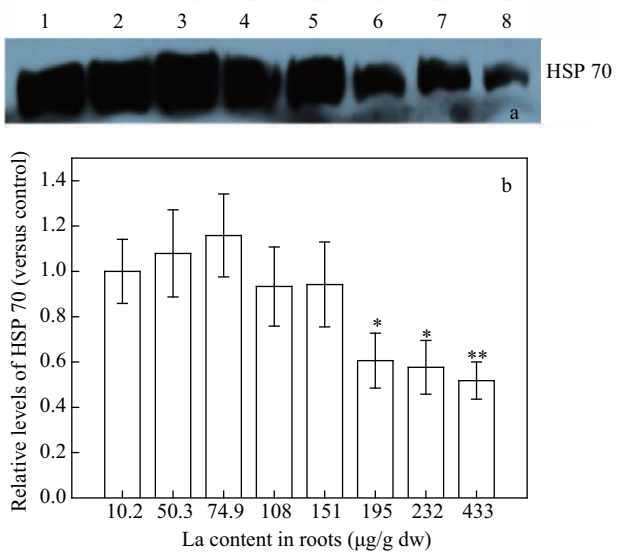

Fig. 5 Western blotting of HSP 70 production (a) and relative HSP 70 levels against controls (b) in roots of *V. faba* seedlings hydroponically cultivated in 0–12 mg/L of extraneous La for 15 days. Lanes 1–8 represent 10.2, 50.3, 74.9, 108, 151, 195, 232 and 433 μg La/g dw roots, respectively. Data are presented mean ± SD (n = 3). * $p < 0.05$, ** $p < 0.01$.

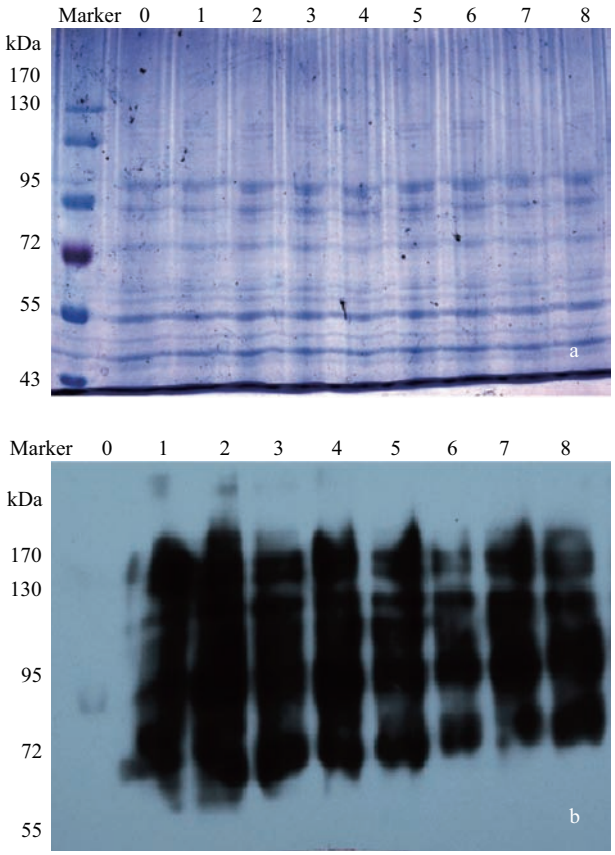

Fig. 6 Immunoblotting analyses of carbonyl proteins in roots of *V. faba* seedlings hydroponically cultivated in 0–12 mg/L of extraneous La for 15 days. (a) total protein stained by Commassie R-250; (b) DNPH-derivatized proteins detected by anti-DNPH antibody. Molecular mass markers were indicated on the left. Lane 0 (blank lane) represents 10.2 μg La/g dw root sample without pretreatment with DNPH. Lanes 1-8 represent 10.2, 50.3, 74.9, 108, 151, 195, 232 and 433 μg La/g dw root samples pretreatment with DNPH, respectively.

in harmful effects (Kool et al., 2007). ROS is known to cause oxidatively modified proteins by the production

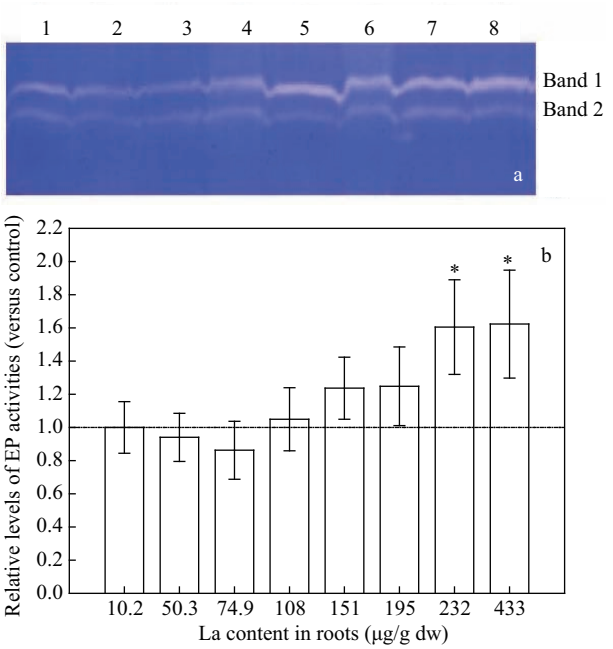

Fig. 7 EP isozymes and activities in roots of *V. faba* seedlings hydroponically cultivated in 0–12 mg/L of extraneous La for 15 days. (a) a representative picture of EP isozymes; (b) relative activities of EP isozymes. Data are presented as mean ± SD (n = 3). * $p < 0.05$. Lanes 1–8 represent 10.2, 50.3, 74.9, 108, 151, 195, 232 and 433 μg La/g dw roots, respectively. A short dash line was introduced in the figure, proposed as a hormetic threshold level, above which represents oxidative stress.

of carbonyl groups in polypeptide molecules, which is considered as a good index of oxidative stress and can be detected by derivatization with DNPH (Levine et al., 1994; Romero-Puertas et al., 2002).

In the present study, the intensities of bands indicating oxidatively modified proteins tended to decrease from 50.3 to 195 μg La/g dw, and then enhanced in the roots (Fig. 6b). This can be attributed to the enhancement of proteolytic degradation at higher concentrations of La in the roots (Fig. 7).

Oxidatively modified proteins are usually selectively recognized and preferentially degraded by intracellular proteolytic enzymes such as EPs and proved to be valid in plants (Solomon et al., 1999). EPs have multiple isozymes in response to elevated proteolysis when plants are subjected to senesce or oxidative stress (Distefano et al., 1997; Romero-Puertas et al., 2002). In this study, the U-shaped dose response curve of EP activities suggest that the enhanced proteolytic activities were responsible for the degradation of oxidatively modified proteins and the reduction of immunoblotted carbonyl proteins at higher concentrations of La (Fig. 6b). Thus, the activation of EP isozymes may be another mechanism of detoxification against La-induced protein damage in the roots.

Evans Blue staining *in situ* can be used as a marker of cell death (Romero-Puertas et al., 2004b). As shown in Fig. 2b, cell death was induced in the root tips when the La contents were more than 151 μg/g dw in the roots. Remarkably, at 50.3 μg La/g dw, the root lengths decreased to be the shortest in all the treatments except at 433 μg La/g dw (Fig. 2a). This may be attributed to the much more accumulation of carbonyl proteins and lower activities

of EP isozymes at this treatment in the roots (Fig. 6b, Fig. 7). However, the detected carbonyl proteins did not significantly increase along with the increase of La (Fig. 6b). This may be due to the activation of EP isozymes and subsequently enhanced degradation of carbonyl proteins, resulting in reduction of components of intracellular proteins, increment of cell death, and thus retardation of cell growth in the roots.

In the present study, the increasing concentrations of La caused a series of U-shaped, inverted U-shaped or consecutively biphasic dose response curves in multiple physiological parameters, indicative of hormetic effects. To define the hormetic threshold level of a pollutant, short dash line was firstly introduced into histogram in a previous report (Wang et al., 2010). A positive or beneficial effect usually appears at lower concentrations of pollutants, and may be proposed to occur below a hormetic threshold level. In contrast, an adverse or toxic effect occurs above the hormetic threshold level at higher doses. Similarly, such lines were helpful to delimit the threshold dose range of La in the culture solution (Figs. 4 and 7).

As shown in Fig. 4, based on the short dash lines, the hormetic threshold dose indicating an initial oxidative stress can be defined as 195 μg La/g dw in the roots. Whereas, the relative activities of SOD and APX isozymes were significantly enhanced at more than 232 μg La/g dw, and CAT activities at 433 μg La/g dw compared with the controls, respectively. It suggests that the traditional threshold dose of La contents in the roots ought to be defined as 232 μg La/g dw. By combination of both of the threshold doses, their threshold dose range of La contents may be firstly defined as 195–232 μg La/g dw in the roots, corresponding to 4–8 mg/L of extraneous La in the culture solution based on the activities of antioxidant enzymes. Similarly, by the combination of EP activities and HSP 70 production, their threshold dose range may be secondly delimited as 108–195 μg La/g dw, corresponding to 1–4 mg/L of extraneous La (Figs. 5 and 7).

Until recently, REEs have not been characterized as essential elements and environmentally hazardous metals to plants. The present results confirmed that no less than 0.90 mg/L of soluble La may lead to oxidative stress in the roots of *V. faba* seedlings.

4 Conclusions

This study demonstrated that the increase of La contents in culture solutions and roots led to the enhancement of antioxidant isozymes, EP isozymes and HSP 70 production, constituting defense and detoxification systems against La-induced oxidative stress. Oxidatively modified proteins were most possibly involved in cell death and subsequent growth delay in the roots. The concentrations of La induced a series of hormetic dose responses of SOD, CAT, GPX, APX and EP activities, as well as HSP 70 production in the roots. The threshold dose range was firstly proposed as 108–195 μg La/g dw in the roots, corresponding to 0.90–3.12 mg/L of soluble La and 1–4 mg/L of extraneous La in the culture solution. These results imply that persistent applications of REEs may lead to potential ecological risk in the environment in the future.

Acknowledgments

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